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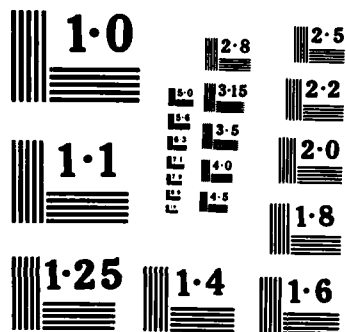   INFECTIOUS DISEASES FORT DETRICK MD
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The presence of insulin receptors was investigated in human Y-79 retinoblastoma cells grown in suspension culture. The binding of $[^{125}I]$ insulin to these cells was time-, temperature-, and pH-dependent, was competed for by insulin and proinsulin but not other peptides, and was inhibited by antibodies against the insulin receptor. The Scatchard plot of insulin competition data was curvilinear and was resolved in a high affinity ($K_d \sim 0.5 \times 10^{-9}$ M) - low capacity

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(~ 3,000 sites/cell) and a low affinity (K_d ~ 1 x 10^{-7} M) – high capacity (~ 155,000 sites/cell) component. Negative cooperativity was not found, in agreement with other studies in rodent neural cells. However, in contrast to studies with rodent cells, insulin specifically down-regulated its receptor on human Y-79 cells after prolonged exposure.

In conclusion, these data show for the first time the presence of specific insulin receptors in human Y-79 retinoblastoma cells. As these cells were previously shown to have several characteristics typical of neural cells, we propose their use as a model to study the effects of insulin on neural and retinal tissues of human origin.

HUMAN Y-79 RETINOBLASTOMA CELLS
EXHIBIT SPECIFIC INSULIN RECEPTORS

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Abbreviations used: NGF: nerve growth factor; hGH: human growth hormone;

FGF: fibroblast growth factor

ABSTRACT

The presence of insulin receptors was investigated in human Y-79 retino-
blastoma cells grown in suspension culture. The binding of [^{125}I] insulin to
these cells was time-, temperature-, and pH-dependent, was competed for by
insulin and proinsulin but not other peptides, and was inhibited by antibodies
against the insulin receptor. The Scatchard plot of insulin competition data
was curvilinear and was resolved in a high affinity ($K_d \approx 0.5 \times 10^{-9}$ M) - low
capacity ($\approx$ 3,000 sites/cell) and a low affinity ($K_d \approx 1 \times 10^{-7}$ M) - high
capacity ($\approx$ 155,000 sites/cell) component. Negative cooperativity was not
found, in agreement with other studies in rodent neural cells. However, in
contrast to studies with rodent cells, insulin specifically down-regulated its
receptor on human Y-79 cells after prolonged exposure.

In conclusion, these data show for the first time the presence of
specific insulin receptors in human Y-79 retinoblastoma cells. As these cells
were previously shown to have several characteristics typical of neural cells,
we propose their use as a model to study the effects of insulin on neural and
retinal tissues of human origin.

Key Words: Insulin receptor, retinoblastoma

Running Title: Insulin receptors in retinoblastoma cells

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Several studies have recently demonstrated that insulin and insulin receptors are widely distributed in the central nervous system, including the retina, of many animal species and man (Havrankova et al 1978 a, b; Dorn et al 1982; Landau et al 1983; Sara et al 1983; Raizada 1983; Das et al 1984, 1985; DiMattio and DiPaolo 1985; for reviews see Van Houten and Posner 1981; Underhill et al 1982). These findings have raised questions about the biological role(s) of insulin in the CNS. The functions, however, of insulin in the brain remain unclear (Daniel et al 1977; Szabo and Szabo 1982; Underhill et al 1982).

Since the first step in the action of insulin is binding to its receptor (Ginsberg 1977), recent studies have focused on characterization of the properties and regulation of the insulin receptor in nervous tissue. Almost all studies to date have been conducted with rodent brain membranes (Havrankova et al 1978a, 1979; Yip et al 1980; Heidenreich et al 1983; Rees-Jones et al 1984) or rodent primary nerve cell cultures (Raizada et al 1980, 1982; Boyd and Raizada 1983; Clarke et al 1984; Van Schravendijk et al 1984), with no comparable studies in neural cells of human origin. We, therefore, explored the human Y-79 retinoblastoma cell line (Reid et al 1974) for the presence of insulin receptors. These cells were recently shown by us (Kyritsis et al 1984 a, b, c; Madtes et al 1985) to differentiate into cells having several morphologic, histochemical, and biochemical features of neural cells and thus seemed appropriate for our purpose. We report here our finding of specific insulin receptors in Y-79 retinoblastoma cells and their partial characterization.

MATERIALS AND METHODS

Materials

Porcine insulin and proinsulin were from Elanco Products Co. Nerve growth factor, fibroblast growth factor and porcine glucagon were from Sigma. Human growth hormone was a gift of the National Pituitary Agency, NIADDK, NIH. Serum containing insulin receptor antibodies (Flier et al 1977) was a gift of Dr. Philip Gorden, Diabetes Branch, NIADDK, NIH. Eagle's minimum essential medium supplemented with Earle's salts (EMEM), glutamine, and fetal bovine serum were from Flow Labs. $Na^{125}I$ (carrier-free) was purchased from Amersham Nuclear Co. All other reagents were of analytical grade and obtained from commercial sources.

Culture of Y-79 retinoblastoma cells

Cells were maintained in suspension culture in EMEM containing 10% fetal bovine serum and 2 mM glutamine at 37°C in a humidified incubator with 95% air/5% CO_2. Details of the culture procedures have been published (Reid et al 1974; Madtes et al 1985).

Insulin Iodination

^{125}I labeled porcine insulin was prepared by a modification of the chloramine-T method (Roth 1975) at specific activities of 120-180 μCi/μg and purified by chromatography on a cellulose column. The ^{125}I labeled hormone preparation used in the binding assays was over 98% precipitated by 10% trichloroacetic acid (TCA).

[^{125}I]insulin binding assay

Y-79 cells in early stationary phase were centrifuged at 500 x g for 5 min at 4°C, washed three times with "binding buffer" (Hepes 50 mM, NaCl 120 mM, KCl 5 mM, MgSO$_4$ 1.2 mM, sodium acetate 15 mM, glucose 10 mM, EDTA 1 mM, BSA 1%, pH 7.8) and resuspended in the same buffer at a concentration of 6-8 x 10^6 cells/ml. Cell viability was determined by the trypan blue method (Phillips 1973). Ninety per cent or more of the cells excluded the dye. Cells (400 µl) were incubated in a final volume of 0.5 ml with [^{125}I]insulin alone (0.1 ng/ml) or with various concentrations of unlabeled insulin (1-10^5 ng/ml), usually at 22°C for 90 min, except as indicated in figure 1 (one). At the end of the incubation, 400 µl aliquots were transferred into chilled 1.5 ml plastic microfuge tubes containing 1.0 ml chilled binding buffer and centrifuged for 1.5 min in a Beckman type B microfuge. The supernatant was aspirated and discarded, and the tips of the tubes, containing the cell pellets, were excised and counted in a gamma spectrophotometer with 77% efficiency. Specific binding was calculated by subtracting the counts associated with cell pellets in the presence of 100 µg/ml unlabeled insulin (non-specific binding, NSB) from the counts associated with the other cell pellets. Degradation of unbound labeled hormone in the supernatant was assessed by precipitation with 10% TCA.

Analysis of binding-competition data was done by a version of the program LIGAND (Munson and Rodbard 1980) for the Apple IIe computer (Biomedical Computing Technology Information Center, Nashville, TN).

Dissociation of bound [^{125}I]insulin

Cells were preincubated with [^{125}I]insulin for 90 min at 22°C in a final volume of 500 μl. Aliquots (10 μl) of unlabeled insulin or other test peptides were then added to a final concentration of 10 μg/ml, and incubation was continued for various periods of time. Cell-associated radioactivity was determined as described above.

Dissociation experiments designed to test for negative cooperativity were performed essentially as described (DeMeyts et al 1976). Briefly, a batch of Y-79 cells was incubated with tracer amounts of [^{125}I]insulin (0.2 ng/ml) for 90 min at 22°C. Then cells were centrifuged (800 x g for 2 min at 4°C), resuspended in binding buffer in the original volume, and aliquots (0.1 ml) were transferred to tubes containing 10 ml of binding buffer, with or without the presence of unlabeled insulin (1 μg/ml). Cell-bound radioactivity was measured at different time intervals and expressed as a percentage of binding at the beginning of dissociation. Non-specific binding was determined in a batch of cells treated as above but incubated with insulin (10 μg/ml) during the association phase.

Effect of insulin receptor antibody on [^{125}I]insulin binding

The effect of human serum containing insulin receptor antibodies was evaluated as was previously described (Flier et al 1977). Briefly, cells were preincubated with various dilutions of serum for 60 min at 37°C, washed five times with ice cold buffer with a 10 min incubation period at 4°C between washes, and were finally used in a standard binding assay as described above. Immune human serum not containing insulin receptor antibodies or buffer containing insulin (50 ng/ml) were used as controls. Data are expressed as percent of binding obtained in cells incubated with buffer alone.

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Effect of preincubation with insulin on subsequent [^{125}I]insulin binding

Cells at early stationary phase were washed twice with culture medium and resuspended in the original concentration in culture medium containing various amounts of unlabeled insulin. After 18 hours at 37°C, cells were washed extensively (5x) with culture medium to remove added insulin, resuspended in binding buffer, and incubated with [^{125}I]insulin. Total and non-specific binding were determined as described above. Data are expressed as percent of binding obtained in cells without pre-exposure to hormone.

[^{125}I]insulin-binding characteristics of Y-79 retinoblastoma cells

The association of [^{125}I]insulin (0.1 ng/ml) with Y-79 retinoblastoma

cells (5 x 10^6/ml) was a time- and temperature-dependent process (Fig. 1).

Specific binding achieved equilibrium by 30 min, 60 min, and 16 hours at 37°C,

22°C, and 4°C, respectively. Approximately the same amount of labeled hormone

bound specifically at 22° and 4°C, but less at 37°C. Non-specific binding,

obtained in the presence of excess unlabeled hormone (100 μg/ml), was about

60% of total binding at 37°C, 15% at 22°C, and <5% at 4°C. Degradation

of [^{125}I]insulin, assessed in the supernatant with 10% TCA, was low (<5%) at

22°C or 4°C, but it was over 30% at 37°C. For this reason, all subsequent

studies were performed at 22°C for 90 min. Under these conditions [^{125}I]

insulin binding was a linear function of cell concentration from 2 x 10^6 to 1

x 10^7 cells/ml (not shown).

[^{125}I]insulin binding depended on the pH of incubation buffer and was

optimal at pH 7.8 (Fig. 2).

The dissociation of [^{125}I]insulin bound to cells at equilibrium was rapid

after addition of unlabeled insulin (10 μg/ml final) (Fig 3A). About 60-70%

of bound radioactivity dissociated under these conditions after 2 hours, and

70% of this did so within the first 15 min. In contrast to insulin, glucagon,

and nerve growth factor (NGF) had no effect on the dissociation of prebound

insulin.

Dissociation of cell-bound [^{125}I]insulin was also performed after removal

of the unbound labeled hormone by mild centrifugation, resuspension of cells

in the original volume, followed by dilution in buffer with or without

addition of unlabeled insulin (1 μg/ml) at 22°C (Fig 3B). This experiment,

performed to test for cooperative interactions among insulin receptors, showed that the rate of dissociation of [^{125}I]insulin was virtually the same in the presence or absence of unlabeled hormone. As in dissociation without dilution (Fig 3A), a significant portion (about 40%) of the hormone could not be dissociated, and most of the dissociable hormone was released in the early phase of dissociation (Fig 3B).

To further characterize the insulin binding site, we performed binding assays over a wide range (1- 10^5 ng/ml) of unlabeled insulin (Fig 4). A broad displacement curve was obtained, and the concentration of unlabeled insulin inhibiting binding by 50% (ID_{50}) was between 1-5 ng/ml. Presentation of these data in a Scatchard plot ([^{125}I]insulin bound/free vs concentration of bound insulin) (Scatchard 1949) gave a concave upward curve (Fig. 5). In the absence of negative cooperativity (see above), the shape of this curve suggested the presence of binding sites with varying affinities for insulin. We obtained approximate estimates of binding constants using the computer program LIGAND. Best fit was obtained by a two-site model plus non-specific binding. The high-affinity binding site had a dissociation constant (k_d) of approximately 0.5×10^{-9} M and a binding capacity (R) of 2.6×10^{-11} M ($\sim$ 3,000 sites/cell). The other site had a k_d of approximately 1×10^{-7} M and R of 1.3×10^{-9} M ($\sim$ 155,000 sites/cell).

Specificity of [^{125}I]insulin binding

Specificity of the observed binding sites for insulin was evaluated by measuring the effect of various peptides and of serum which contained antibodies against the insulin receptor on [^{125}I]insulin binding.

Proinsulin competed for [^{125}I]insulin binding as well as insulin (Fig 4), but the ID_{50} of proinsulin (90 ng/ml) was higher than that for insulin as

expected for this less active precursor of insulin. On the other hand, other peptides such as glucagon, human growth hormone (hGH), fibroblast growth factor (FGF), and NGF, could not compete for labeled insulin binding, even at high concentrations.

When cells were pre-exposed (37°C, 60 min) to human serum containing insulin receptor antibodies and [^{125}I]insulin binding was then measured, a progressive decrease of binding was observed (Fig 6). Inhibition was almost complete at serum dilution 1:10 and half-maximal at 1:60. Pre-exposure of cells to control immune human serum or insulin at 50 ng/ml under similar conditions had no inhibitory effect on insulin binding.

Insulin effect on [^{125}I]insulin binding

Cells were incubated in culture medium in the presence of various concentrations of unlabeled insulin (10^1 - 10^4 ng/ml) for various time periods at 37°C, washed, and then incubated with labeled insulin. There was a dose-dependent decrease in insulin binding (Fig 7) after pre-exposure to insulin but not to other peptides. Minimal effects of insulin could be observed as early as 4 hours (not shown) with maximal inhibition present at 18 hours.

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FIGURE LEGENDS

Legend for Figure 1

Time course of [^{125}I]insulin binding to retinoblastoma cells. Stationary cells in suspension culture were incubated with [^{125}I]insulin (100 pg/ml) at 4°C (♦,◊), 22°C (●, o) or 37°C (■,□) in the absence (filled symbols) or presence (open symbols) of unlabeled insulin (100 µg/ml). Specific binding, shown as a continuous line, was determined as described in Materials and Methods.

Legend for Figure 2

pH Dependence of [^{125}I]insulin binding. Cells were incubated with [^{125}I]insulin for 90 min at 22°C in assay buffer with pH varying from 6.8 to 8.2 and cell-bound radioactivity was determined as described previously.

Legend for Figure 3

Dissociation kinetics of bound [^{125}I]insulin. Cells were incubated to equilibrium with [^{125}I]insulin (100 pg/ml) at 22°C for 90 min prior to dissociation experiments. (A) Dissociation patterns without dilution of incubation medium after addition of excess unlabeled peptide (10 µg/ml): (●) insulin; (■) nerve growth factor; (▲) glucagon. (B) Dissociation patterns after centrifugation of cells to remove free [^{125}I]insulin followed by 100-fold dilution in the presence (●) or absence (o) of unlabeled insulin (1 µg/ml). Each point represents the mean ± SE of three experiments performed in triplicate.

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1983) and differentiation, including induction of synaptic functions (Kessler et al 1984; Puro and Agardh 1983), and modulate neurotransmission (Palovick et al 1984; Rhoads et al 1984).

In conclusion, we have found specific insulin receptors on human Y-79 retinoblastoma cells which resemble those present in peripheral cells. Our present observations, and our recent ability to induce differentiation of these cells into neural cell types (Kyritsis et al 1984a, b) suggest that the Y-79 retinoblastoma cells could be used as a model to study the insulin receptor and its function in human neural and retinal tissues.

evidence for an insulin receptor subtype in CNS cells; this is clearly not the case in Y-79 cells, although structural studies of the receptor will be required to evaluate this point. It has been stated that glial but not neuronal insulin receptors are down-regulated by insulin (Boyd and Raizada 1983). Therefore, the undifferentiated Y-79 cell could be exhibiting a glial-type response. Further studies with differentiated retinoblastoma cells could resolve this issue. Since ligand-induced receptor down-regulation requires protein synthesis (Kosmakos and Roth 1980) and may play a role in regulation of cell sensitivity to the hormone (Ginsberg 1977) our finding suggests the presence of this pathway of insulin action on Y-79 cells.

The functional role of insulin in Y-79 cells is not yet known. Except for insulin-induced down-regulation of the insulin receptor, we have been unable to observe other effects of insulin, such as stimulation of glucose and amino acid transport or a decrease in catecholamine-stimulated cyclic AMP levels (results not shown). Other studies with freshly isolated brain (Gorus et al 1984) or retinal cells (DiMattio and DiPaola 1985) also failed to show insulin effects on glucose uptake or metabolism. This, however, could be due to cellular heterogeneity, since glucose uptake was recently shown to respond to insulin in glial cells but not in mixed neural cell cultures (Clarke et al 1984). Since this is not a problem in our studies, the most likely explanation for our findings is uncoupling of the insulin receptor from effector systems for the studied bioeffects. Insulin, however, could mediate other effects on neural cells and tissues, such as stimulation of protein phosphorylation (Rees-Jones et al 1984; Akers and Routtenberg 1984) and Na^+/K^+ ATPase activity (Bernstein et al 1981), and changes in membrane ionic conductance (Moore 1983). It may also affect neural growth (Yang and Fellows 1980; Van Schravendijk 1984; Yang et al 1981; Raizada et al 1980; Sara et al

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We observed that dissociation of bound [^{125}I]insulin after dilution was not accelerated in the presence of unlabeled insulin, and it was virtually the same as dissociation without prior dilution. This observation, which suggests the absence of negative cooperative interactions among insulin receptors on Y-79 cells (DeMeyts et al 1976; Ginsberg 1977), has also been made in brain cells from mice (Van Schravendijk et al 1984), but not in chick retina cells (Thomopoulos and Pessac 1979). It has also been observed in some, but not all, non-neural cell types (for references see Van Schravendkijk et al 1984). Whether absence of this phenomenon indicates the presence of heterogeneous binding sites, formation of high-affinity insulin-receptor complexes, structural differences in CNS receptors, such as the recently described altered carbohydrate content and size in rat brain insulin receptor subunits (Heidenreich et al 1983; Yip et al 1980), or other membrane differences between Y-79 and other cells remains to be determined. In the absence of negative cooperativity, we analyzed the binding-inhibition data using a model with two binding sites. The apparent binding constant of the high-affinity site (k_d ~ 0.5 x 10^{-9} M) and the ratio of the binding capacities of the two sites (high/low ~ 1/50) are in the same range as that reported in other neural and peripheral cells.

The ability of insulin to down-regulate its receptors after prolonged exposure, as we observed in Y-79 cells, is a characteristic insulin effect in most, but not all, peripheral cell types studied to date (Van Schravendijk et al 1984). It has not, however, been observed in studies with freshly isolated neural rodent cells where, if anything, insulin up-regulated its receptor (Boyd and Raizada 1983; Van Schravendijk 1984). The reason for these differences among cell types is not known. The absence of insulin-induced receptor down regulation in rodent neural cells was taken as supportive

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DISCUSSION

Our present data definitively show that human Y-79 retinoblastoma cells have receptors for insulin. The specificity of detected $[^{125}I]$insulin-binding sites as true insulin receptors was established by several criteria: (a) the ability of unlabelled insulin to compete for binding of labelled hormone at low concentrations ($ID_{50} \sim 5$ ng/ml), whereas proinsulin, the less active precursor of insulin competed at higher concentrations ($ID_{50} \sim 90$ ng/ml), as expected (Ginsberg 1977); (b) the lack of effect of other hormones or peptides on $[^{125}I]$insulin binding; (c) the ability of insulin but not other peptides to accelerate the dissociation of prebound $[^{125}I]$insulin; and (d) the ability of serum containing antibodies against the insulin receptor (Flier et al 1977) to inhibit insulin binding.

The physicochemical characteristics of the reaction of $[^{125}I]$insulin with Y-79 cells are similar to those previously reported for insulin receptors in several peripheral (non-neural) cells (Ginsberg 1977) and in cultured neural cells from mouse (Van Schravendijk et al 1984) and rat (Raizada et al 1980, 1982; Boyd and Raizada 1983; Clarke et al 1984) brains, and chicken retina (Thomopoulos and Pessac 1979). Thus, binding was found to be time-and temperature-dependent and exhibited appropriate pH optimum. In agreement with several other studies, dissociation of bound labeled insulin was also only partially reversible. This phenomenon was not investigated further; it has been attributed to endocytosis of the hormone (Gorden et al 1980), formation of a high-affinity receptor state (Van Schravendijk et al 1984), including formation of covalent insulin-receptor complexes (Saviolakis et al 1981; Clark and Harrison 1982) or combinations of the above.

Legend for Figure 4

Competition of unlabeled peptide hormones with [^{125}I]insulin for binding
to retinoblastoma cells. Cells were incubated for 90 min at 22°C with
[^{125}I]insulin (100 pg/ml) alone or in the presence of various concentrations
unlabeled hormone: (●) procine insulin; (o) porcine proinsulin; (+) glucagon;
(x) human growth hormone; (□) nerve growth factor; (Δ) fibroblast growth
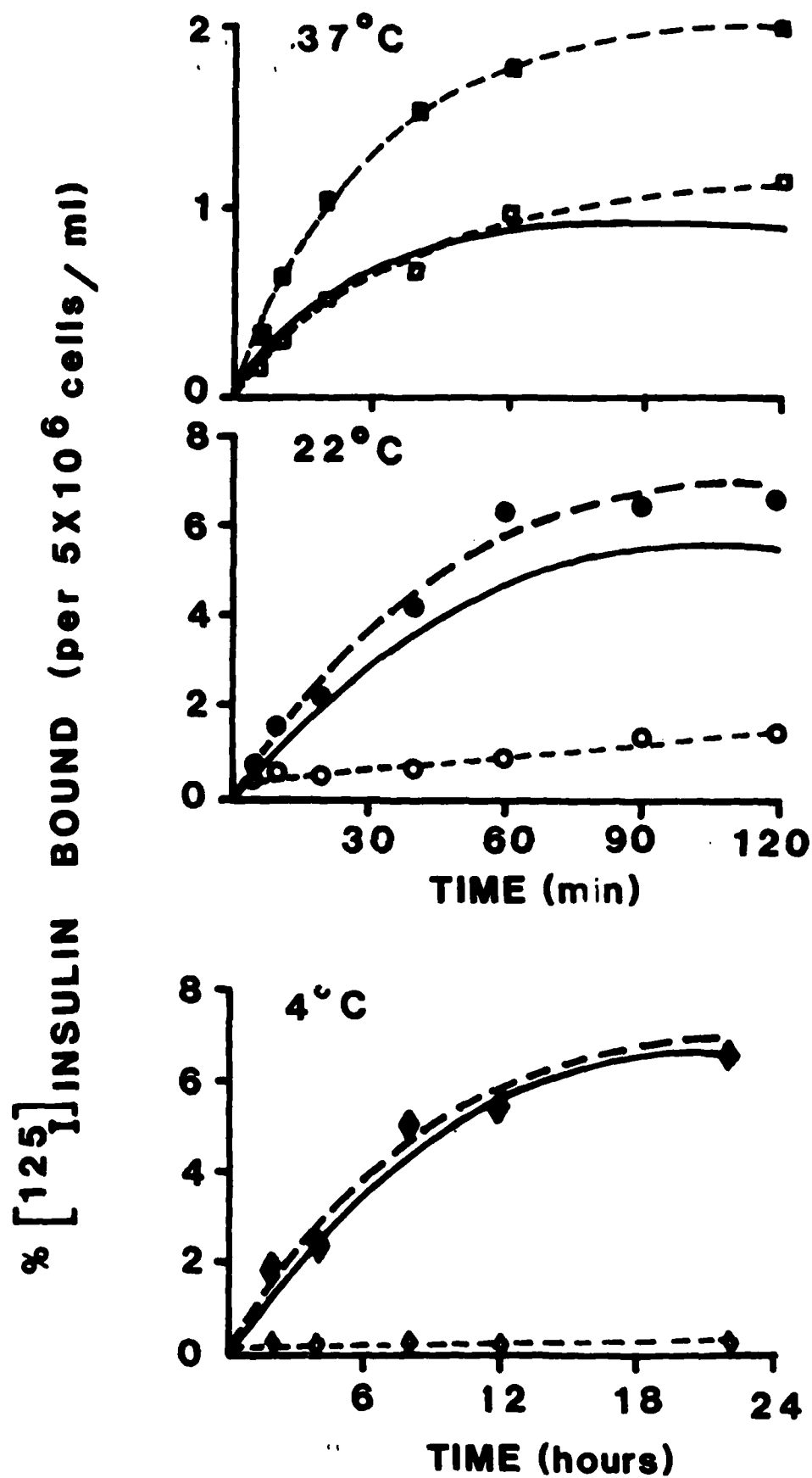factor. Total binding is depicted in this figure.

Legend for Figure 5

Scatchard plot of binding competition between [^{125}I]insulin and unlabeled
insulin. Data are derived from Fig 4 after correction for non-specific
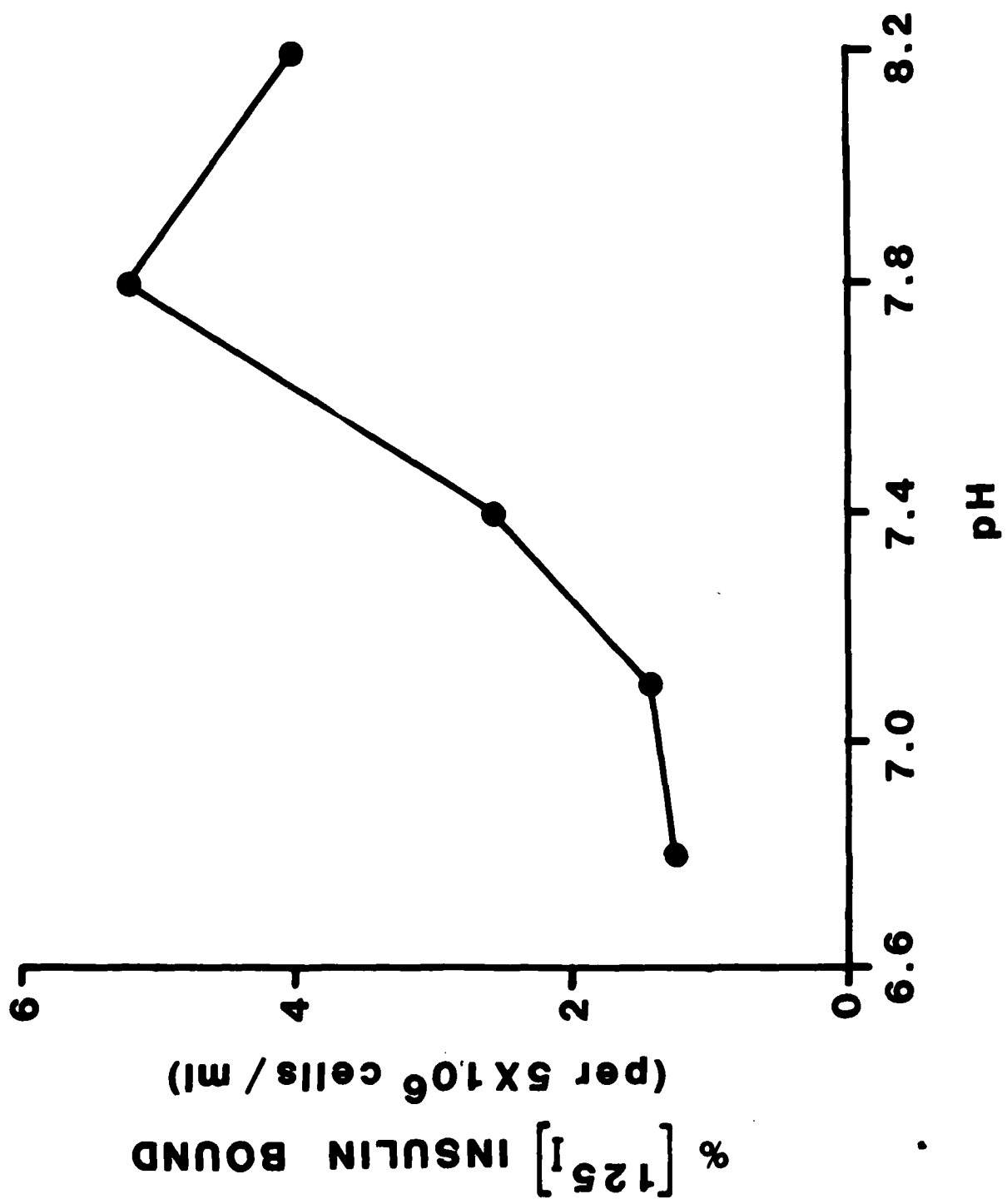binding. Binding parameters obtained by computer analysis are shown in the
inset.

Legend for Figure 6

Inhibition of [^{125}I]insulin binding by serum containing insulin-receptor
antibody. Cells were preincubated at 37°C for 60 min, in the absence or
presence of various dilutions of human serum containing antibody against the
insulin receptor, washed extensively, and used in the standard insulin binding
assay. Control incubations were performed as described in Materials and
Methods. Each point represents the mean ± SE of two experiments performed in
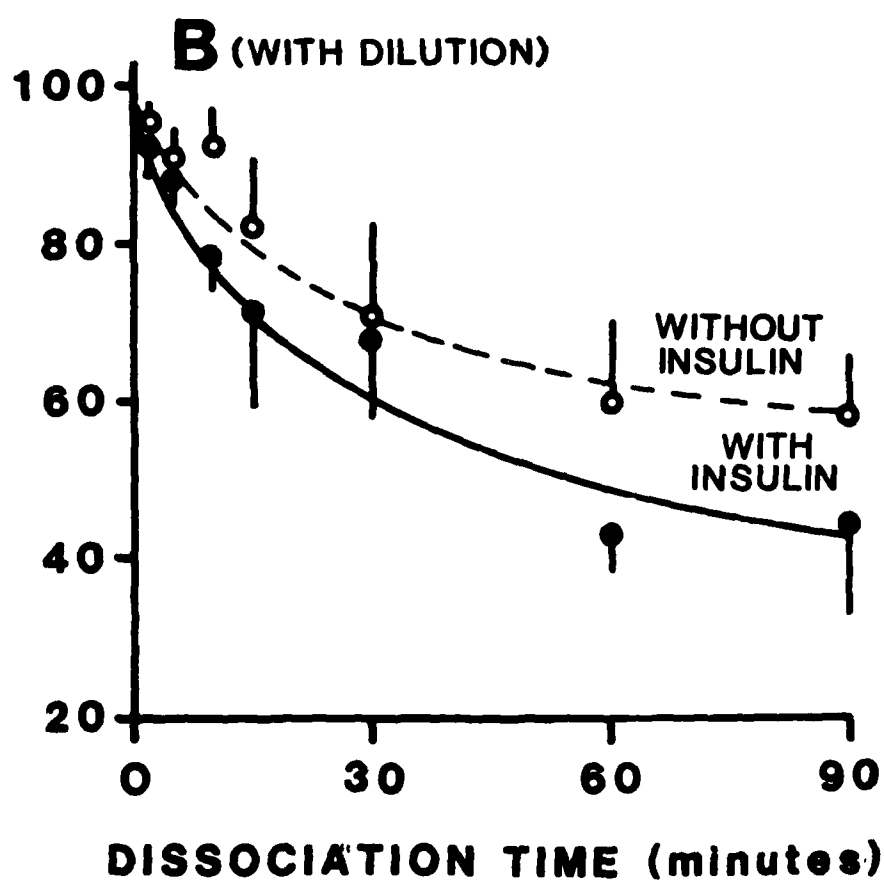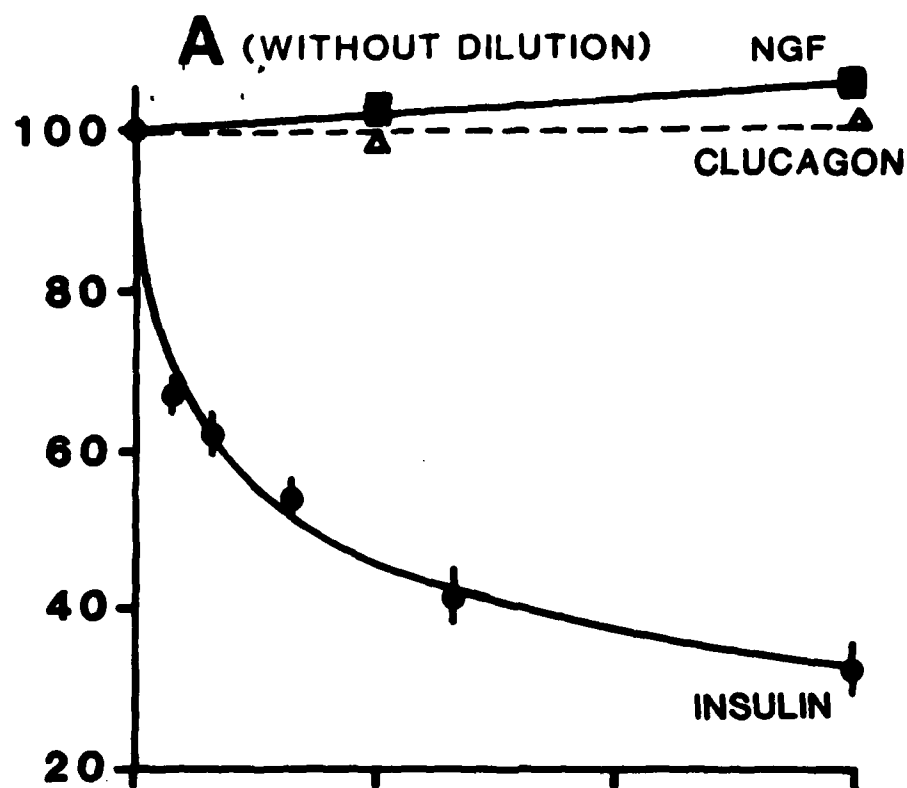triplicate.
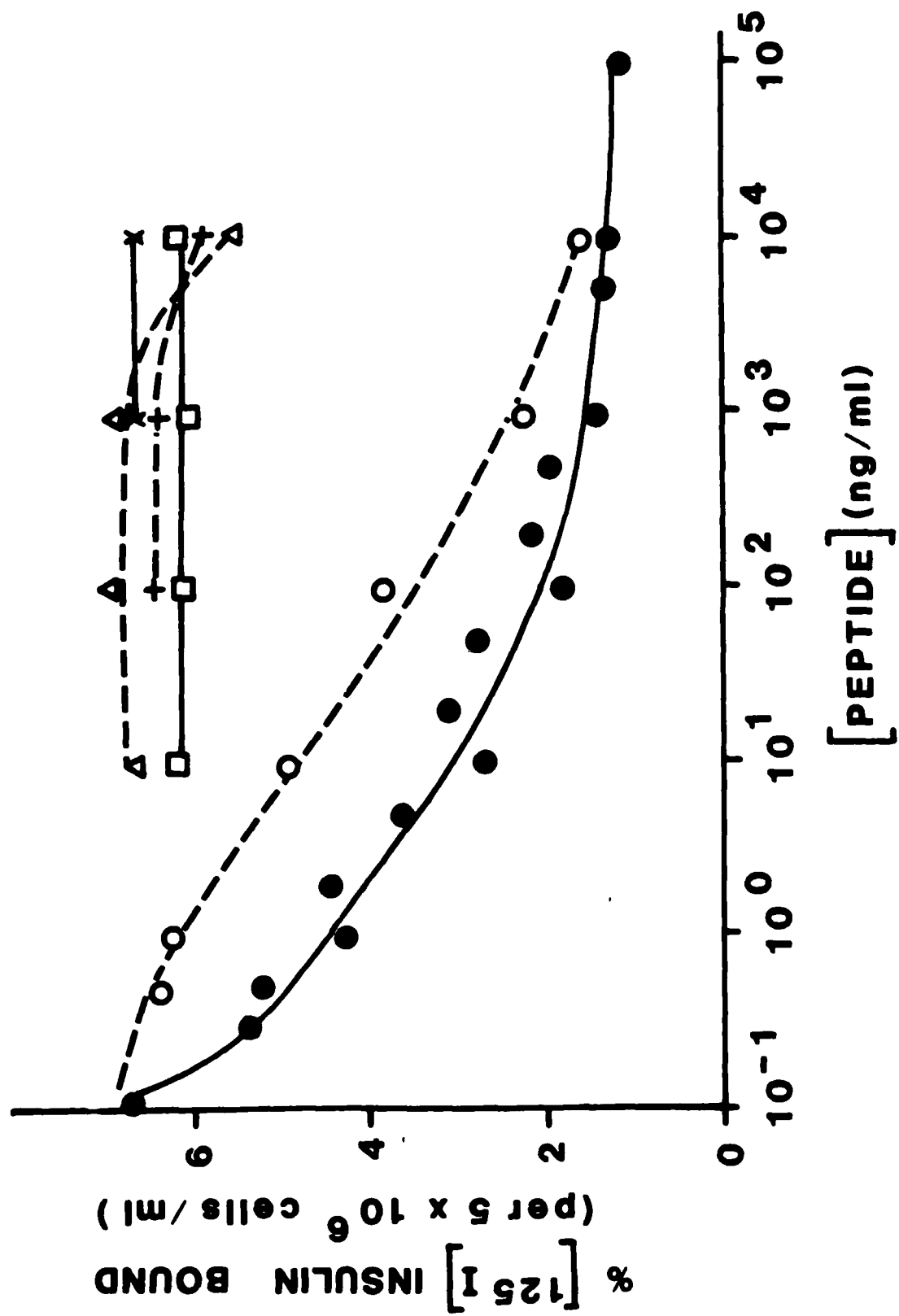
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Legend for Figure 7

Effect of insulin or other peptides on $[^{125}I]$insulin binding to retinoblastoma cells. Cells were preincubated in the absence or presence of various concentrations of unlabeled insulin or other peptide hormones for 18 hours at 37°C, washed extensively, and used in the standard insulin binding assay. Means ± SE of four (insulin) or two (other peptides) experiments performed in duplicate are shown.
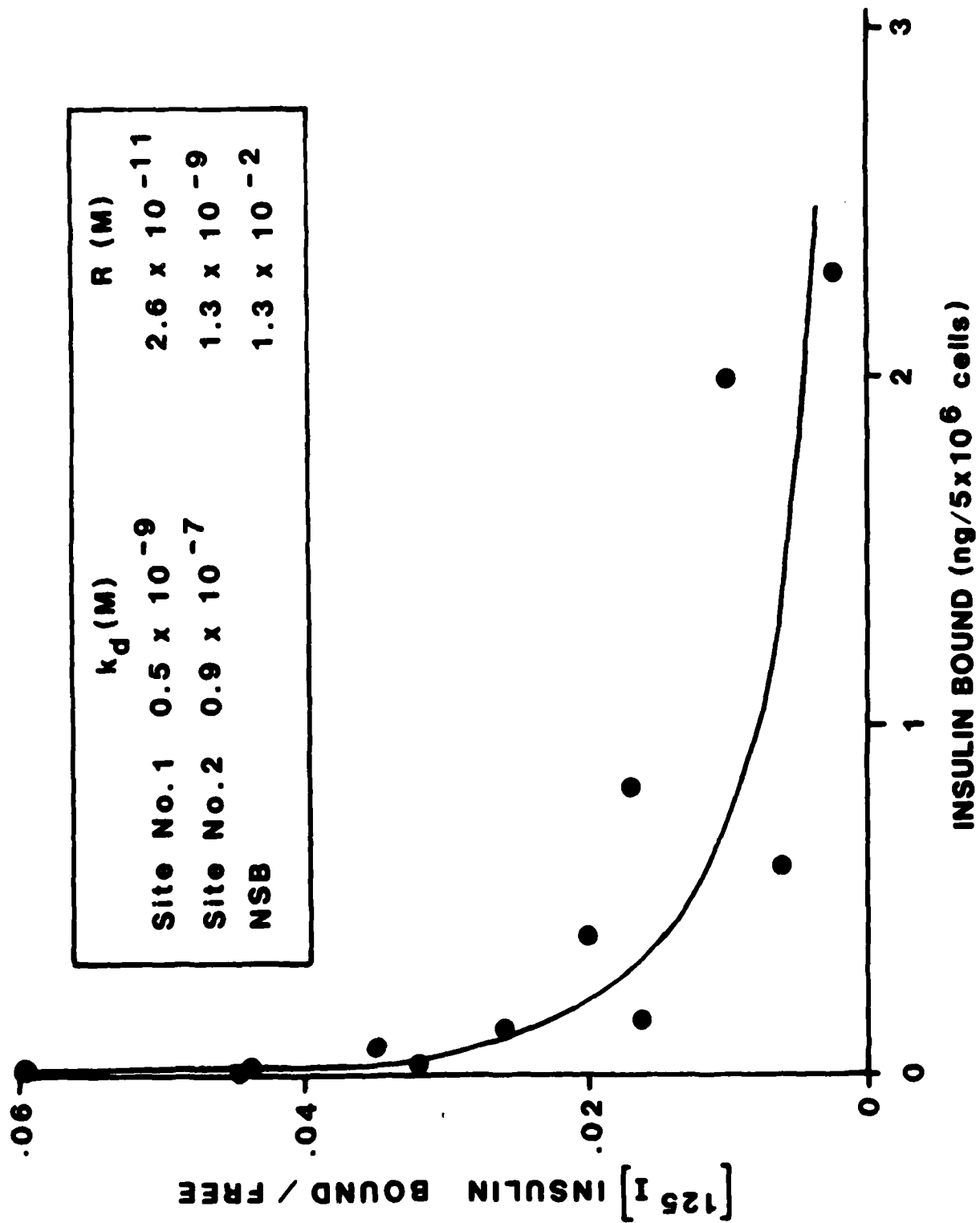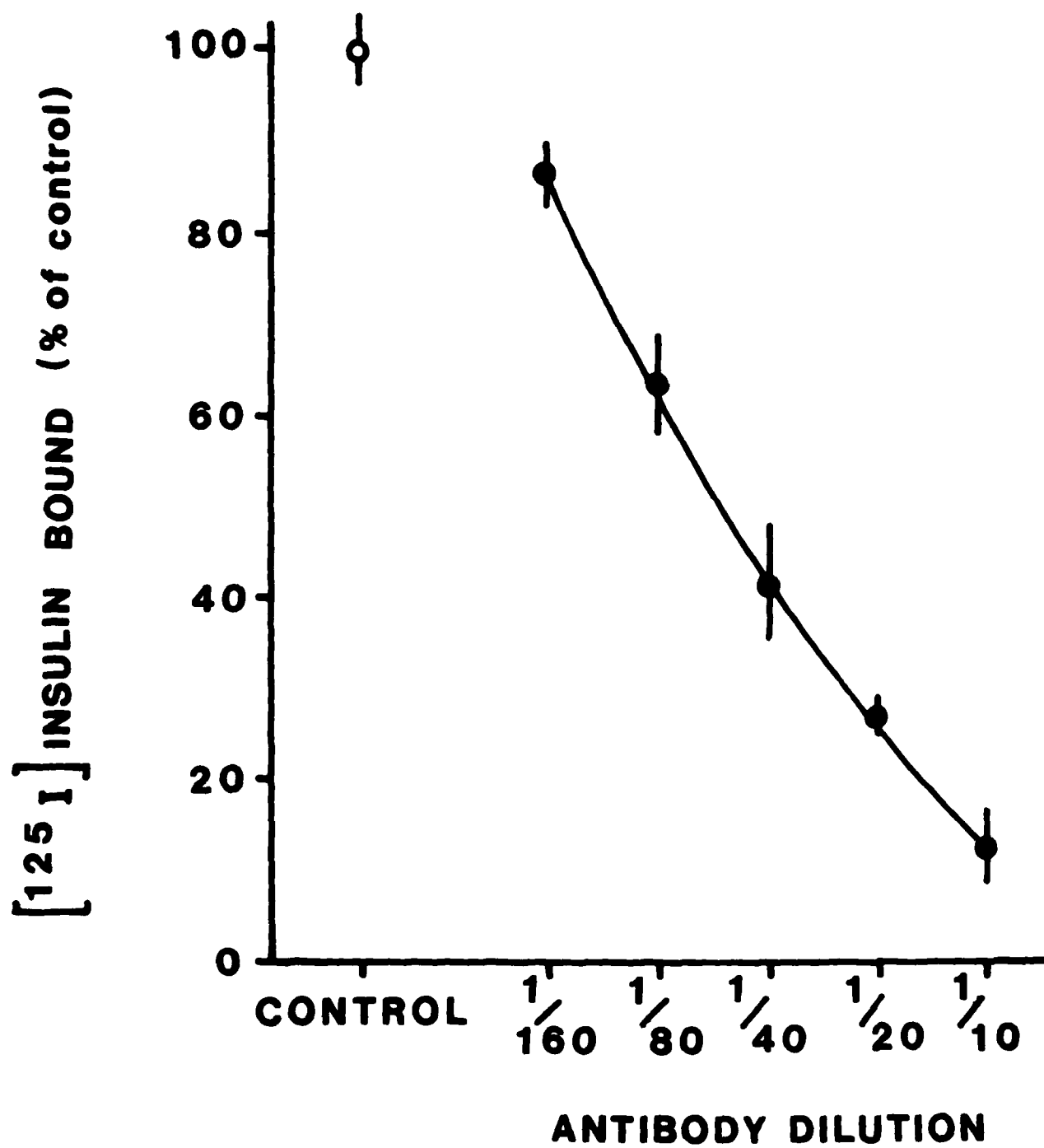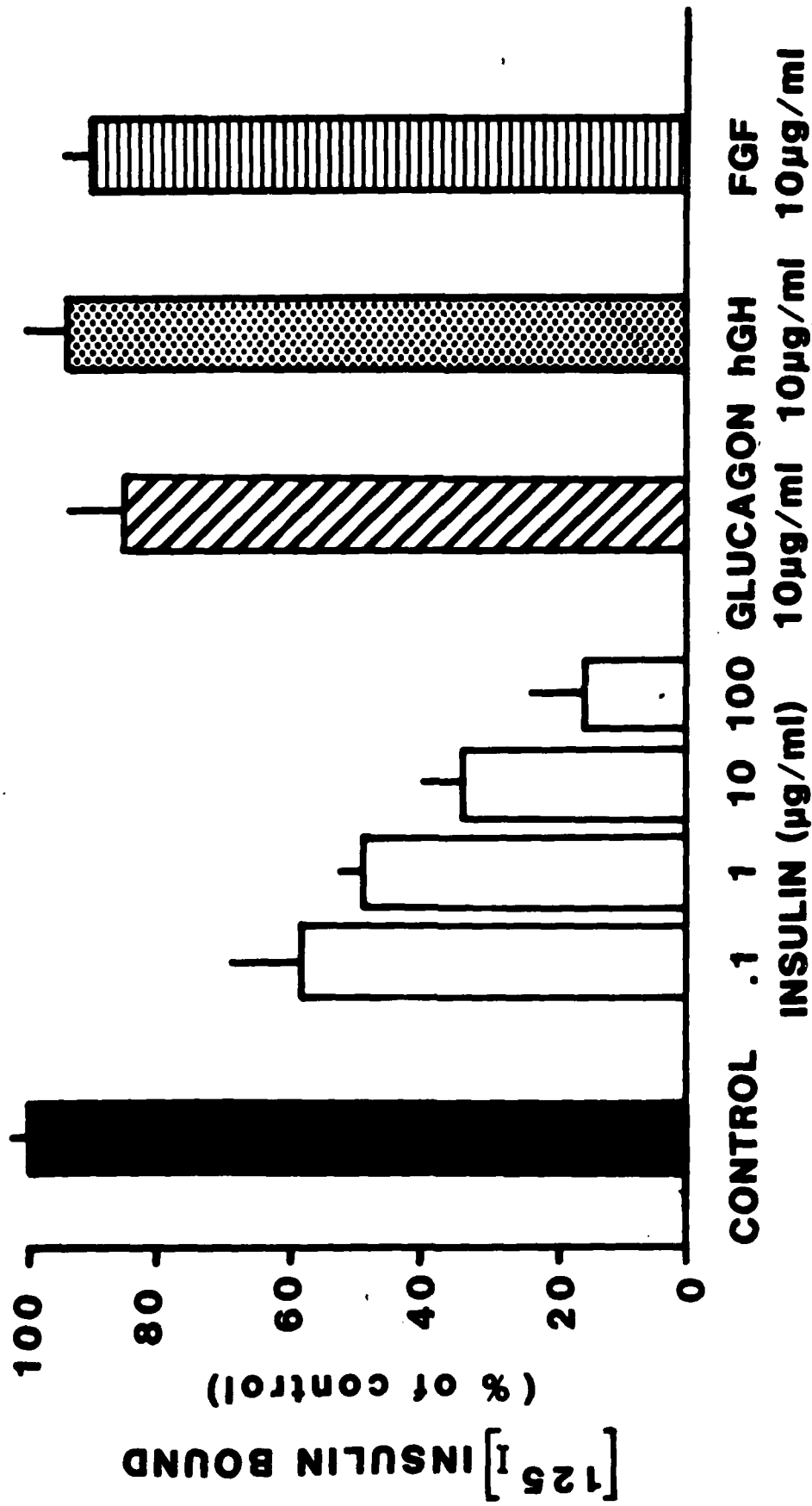
A (WITHOUT DILUTION)

NGF

CLUCAGON

INSULIN

B (WITH DILUTION)

WITHOUT INSULIN

WITH INSULIN

$[^{125}I]$ INSULIN BOUND (% of control at time 0)

DISSOCIATION TIME (minutes)

	k_d (M)	R (M)
Site No.1	0.5×10^{-9}	2.6×10^{-11}
Site No.2	0.9×10^{-7}	1.3×10^{-9}
NSB		1.3×10^{-2}

INSULIN BOUND (ng/5×10^6 cells)

$[^{125}I]$ INSULIN BOUND / FREE

END

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